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ARTICLE

Evidence that trapping of redox-mediators at the surface of *Chlorella vulgaris* leads to error in measurements of cell reducing power.

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The reduction of the redox mediator ferricyanide, $[\text{Fe}(\text{CN})_6]^{3-}$, by a range of algal and bacterial species, is frequently measured to probe plasma membrane ferrireductase activity or to quantify the reducing power of algal/bacterial biofilms and suspensions. In this study we have used rotating disk electrochemistry (RDE) to investigate the reduction of ferricyanide by the model organism *Chlorella vulgaris*. Importantly, we have seen that the diffusion limited current due to the oxidation of ferrocyanide, $[\text{Fe}(\text{CN})_6]^{4-}$, at the electrode decreased linearly as *C. vulgaris* was added to the solution, even though in a pure ferrocyanide solution the algae are not able to reduce the mediator further and are simply spectator ‘particles’. We attribute this effect to trapping of ferrocyanide at the cell surface, with up to 14% of the ferrocyanide missing from the solution at the highest cell concentration. The result has important implications for all techniques that use electrochemistry and other concentration dependent assays (e.g. fluorescence and colourimetry) to monitor ferrocyanide concentrations in the presence of both biofilms and cell suspensions. Analyte trapping could lead to a substantial underestimation of the concentration of reduced product.

Introduction

Iron is an essential trace element which tends to be present in the environment as insoluble Fe(III) oxides.¹ It is thought that the ability of many organisms to reduce chelated iron allows them to solubilise, absorb and utilise iron that would otherwise not be bioavailable. A wide range of bacteria, yeasts, algae and higher plants have the ability to reduce Fe(III) compounds.^{1–6} The reduction process creates solvated Fe(II) that can be transported across the cell membrane and subsequently be re-oxidised for use by the cell. Two pathways for iron uptake are known, strategy I, which occurs in non-vascular plant roots and several algal species including *C. vulgaris*^{7, 8}; and strategy II common to grasses and cyanobacteria.⁹ In strategy I, Fe(III) is reduced by ferric reductases in the plasma membrane, with some studies showing an increase in the concentration of ferric reductase in the membranes of algae grown under iron deficient conditions^{4, 5}. In strategy II, iron uptake is controlled by the release of siderophores; organic ligands that chelate and solubilise Fe(III) allowing it to be transported into cells.⁹ The rate of iron reduction by bacteria and algae is frequently probed using ferricyanide, $[\text{Fe}(\text{CN})_6]^{3-}$, as the electron acceptor. Ferricyanide is useful as the cyano ligands are strongly bound and, unlike many other iron chelates, the molecule does not release free Fe(II) following reduction.^{4, 5} This prevents the reduced iron from crossing the cell membrane and entering the

cell, allowing the total reduction rate to be more easily quantified by solution based techniques. Ferrocyanide has the added benefit of being stable in air and is not slowly oxidised back to ferricyanide under aerobic conditions. The rate of ferricyanide reduction by bacteria and algae is of interest for a variety of reasons. Ferricyanide assays are used to probe plasma membrane redox processes in bacteria and algae.^{1, 2, 5, 10–13} Several species of algae show very high iron reduction rates, higher than needed for solubilisation of trace elements, and the physiological reasons for the high rates are still not fully understood.¹⁴ In the majority of literature studies the concentration of ferrocyanide is determined colourimetrically. The ferricyanide concentration is either measured directly by UV-Vis spectroscopy, or by the addition of bathophenanthroline disulfonate which forms a highly coloured complex with ferrocyanide that can also be detected by UV-Vis.¹⁵

Electrochemical ferricyanide reduction assays have been developed to measure the biochemical oxygen demand (BOD) of waste water solutions.^{16–21} Studies have been conducted using either pure bacterial cultures, mixed cultures, or most recently, activated sludges. The bacteria oxidise biodegradable organics in the waste water and (in the absence of oxygen) use ferricyanide as their terminal electron acceptor, reducing it to ferrocyanide. The total concentration of ferrocyanide is then

detected chronoamperometrically at an electrode surface. The ferricyanide mediated biochemical oxygen demand assay (FM-BOD) has many advantages over the standard method, not least because results can be obtained in a matter of hours rather than the standard five days.

Ferricyanide mediated toxicity assays have been developed using similar principles to the FM-BOD. Yong *et al.* monitored changes in the rate of ferricyanide reduction by *Escherichia Coli* in the presence of a range of pesticides.²² Catterall *et al.* used the same technique to measure the toxicity of a range of organic and inorganic contaminants.¹⁷ In both cases the amount of ferrocyanide was quantified electrochemically.

In this paper we have investigated both static (chronoamperometric) and dynamic (rotating disk) electrochemical techniques for measuring ferricyanide reduction by the single celled green algae *C. vulgaris*. Importantly, rotating disc electrochemistry (RDE) has also allowed us to measure the solution concentration/diffusion coefficient for the reduced mediator ferrocyanide in the presence of algae. It was found that the diffusion limited current is strongly dependent on cell concentration even when the algal cells are simply present as spectator particles. The result has implications for all electrochemical measurements made in the presence of cells, as failing to correct for the change in current will lead to an underestimation of the amount of ferrocyanide present.

Experimental

Culturing conditions of *C. vulgaris*

Stock (Crest Lab at the University of Cambridge) was maintained on sterile 2% wt agar plates made from Bold-Basal Medium with 3-fold Nitrogen and Vitamins (3N-BBM+V) media under a 12:12 hour light:dark regime. Liquid cultures were grown in 250 mL flasks containing 100 mL 3N-BBM+V under constant agitation from an orbital shaker, subjected to 12 hour dark/light cycles from a diffused white light source (fluorescent light box placed under the culture flasks, non-heating, from lightboxUK.net). Cell counts were performed under an optical microscope using a haemocytometer (Bright Line™ from Sigma Aldrich), before cells were prepared for experiments by centrifuging for three minutes at 13.2×10^3 rpm, removing supernatant and re-suspending cells in fresh media at desired concentrations. Measurements were carried out on cells in the stationary growth phase.

Electrochemistry

The reduction of ferricyanide by *C. vulgaris* was measured chronoamperometrically (Autolab PGSTAT 12) at 100 mV past the oxidation potential for ferrocyanide. A range of concentrations of algae and ferricyanide were tested; algal concentrations of 7×10^7 and 1.4×10^8 cells mL⁻¹ were chosen along with 1 mmol dm⁻³, 3 mmol dm⁻³, 5 mmol dm⁻³ and 7 mmol dm⁻³ ferricyanide. Experiments were carried out in a Perspex chamber clamped to a fluorine doped tin oxide (FTO) working electrode (area 1.33 cm²), which allowed light to reach

the cells. Once the experiment was running, the sample was illuminated periodically (620 nm LED, 5×10^{-3} W cm⁻²). Background BBM-3N+V media controls were performed in the presence of ferricyanide (1, 3, 5 and 7 mmol dm⁻³) and subtracted. Three repeat measurements were taken, approximately 20 minutes apart.

All rotating disk measurements were carried out with the electrode held 100 mV positive of the oxidation potential for ferrocyanide. Two distinct experiments were carried out. Firstly, *C. vulgaris* was added to a solution containing 100% ferrocyanide and the oxidation of ferrocyanide monitored in the presence of the spectator cells. In the second set of experiments cells were placed in a solution containing 100% ferricyanide and the amount of ferrocyanide produced by the algae as they reduced the ferricyanide was monitored. A Pt working electrode was used (area 0.07 cm²). The second set of experiments involved keeping the ferricyanide concentration constant at 2.78 mmol dm⁻³, before increasing the cell concentration (taking dilutions into account) from 2.40×10^7 , 4.76×10^7 , 7.07×10^7 , 9.34×10^7 to 1.15×10^8 cells mL⁻¹. In further experiments, algal cell concentration was held constant, and the ferricyanide concentration increased from 2.78, 5.41, 7.89, 10.26, 12.50, 16.00, 19.23 to 22.22 mmol dm⁻³. Finally, the ferricyanide concentration was held constant at 22.22 mmol dm⁻³, and cells again increased from 9.26×10^7 , 1.10×10^8 , 1.28×10^8 , 1.45×10^8 , 1.62×10^8 to 1.79×10^8 cells mL⁻¹. At each set of values, the rotation speed of the working electrode was increased between 100-600 rpm. Media controls were performed and subtracted from measurements. Controls in the presence of media and ferricyanide (1, 3, 5, 7 and 10 mmol dm⁻³) were also carried out; as expected background currents in the presence of ferricyanide (absence of algae) at 100mV positive of the oxidation potential were small (see supporting information, figure S1) and ranged from 0.01 to 0.065 μA. As expected, in the absence of algae no detectable increase in oxidation current was seen as the ferricyanide concentration was increased. The limiting current was measured chronoamperometrically, with each experiment taking 3-5 minutes.

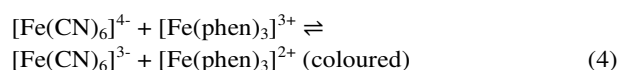
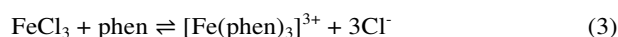
Rotating disc measurements were analysed using either the Koutecky-Levich equation (Equation 1) or the Levich equation (Equation 2). Where i is the current, i_k is the kinetically limited current, i_l is the diffusion limited current, F is Faraday's constant A is the area of the electrode, D is the diffusion coefficient, ω is the rotation speed in radians per second, ν is the kinematic viscosity, n is the number of electrons transferred in the redox reaction and C is the concentration.

$$\frac{1}{i} = \frac{1}{i_k} + \frac{1}{0.62nFAD^{\frac{2}{3}}\omega^{\frac{1}{2}}\nu^{-\frac{1}{6}}C} \quad (1)$$

$$i_l = 0.62nFAD^{\frac{2}{3}}\omega^{\frac{1}{2}}\nu^{-\frac{1}{6}}C \quad (2)$$

Results and Discussion

The most commonly reported method for quantifying the reduction of Fe(III) to Fe(II) by cells (bacterial or algal) is to carry out a colorimetric assay.^{1, 2, 5, 10-12} The appearance of the reduced species (or disappearance of the oxidised species) can be measured directly if the redox mediator absorbs at a convenient wavelength. Ferricyanide, $[\text{Fe}(\text{CN})_6]^{3-}$, shows an absorption maximum at about 420 nm, the disappearance of which can be used to monitor the reduction of ferricyanide to ferrocyanide in real time (see supporting information, Fig. S2). The drawback is that the low extinction coefficient (approx. $1040 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$ at pH 8)⁵ means that this method can lack sufficient sensitivity. To increase the sensitivity, assays for Fe(II) have been developed that involve the addition of ferric ions and a ligand that acts as a ferrous selective chromophore.^{15, 23} Commonly used ligands are o-phenanthroline and batho-phenanthroline (in equations 3 and 4 both are abbreviated to phen).



In the assay the ferric ions are reduced to ferrous ions by the ferrocyanide, $[\text{Fe}(\text{CN})_6]^{4-}$, ultimately forming a highly coloured Fe(II)-phenanthroline complex which is quantified using UV-Vis spectroscopy. Although this method is relatively straightforward it has a number of drawbacks.¹⁵ Firstly, as the $[\text{Fe}(\text{CN})_6]^{4-}$, ferric ions and phenanthroline complex are in chemical equilibrium, if the reaction has not reached completion, then the concentration of phenanthroline complex will not be indicative of the total concentration of $[\text{Fe}(\text{CN})_6]^{4-}$. If the end point is known, the mixture can be incubated until the reaction is complete. This adds to measurement length and requires prior knowledge of the system. Sometimes heating is used to force the reactions to completion, but heating can often damage or change the cells. In addition, it is necessary to carry out the assay in an acidic environment to prevent the inactivation of the ferric ions by hydrolysis. Again, the pH of the environment can substantially change the behaviour of the cells. Finally, due to the chemical equilibria present, the rate of appearance of the coloured complex may not be a direct measure of the rate of $[\text{Fe}(\text{CN})_6]^{4-}$ production. Although the real-time increase in the absorbance of Fe(II) phenanthroline is frequently used to report the rate of $[\text{Fe}(\text{CN})_6]^{4-}$ reduction by cells, it cannot automatically be assumed that a simple correlation can be made.

$[\text{Fe}(\text{CN})_6]^{4-}$ concentrations can be directly and accurately measured using a variety of electrochemical techniques.²⁴ Electrochemical measurements can be carried out quickly at a wide range of pH and no incubation/heating is required. Issues can arise if there are other redox active metabolites in the

solution, although this is only a problem if their redox potential is similar to that of ferrocyanide. A second problem can be the fouling of the electrode surface by biomolecules and steps may need to be taken to functionalise or to regularly clean the active electrode area. One final issue with electrochemical $[\text{Fe}(\text{CN})_6]^{4-}$ determination is that the currents measured are generally diffusion limited (i.e. the rate determining step is the diffusion of ferrocyanide from the cells to the electrode surface).^{6, 16-20} It is therefore possible to determine the total $[\text{Fe}(\text{CN})_6]^{4-}$ concentration in solution at a given time, but not to measure fast enzyme kinetics. $[\text{Fe}(\text{CN})_6]^{3-}$ mediated BOD tests and toxicity tests are carried out by measuring the total $[\text{Fe}(\text{CN})_6]^{4-}$ concentration after a defined incubation time.^{16, 18-20} The steady state diffusion limited current for $[\text{Fe}(\text{CN})_6]^{4-}$ oxidation is measured at a planar disc microelectrode and the concentration of $[\text{Fe}(\text{CN})_6]^{4-}$ in solution is calculated using equation 5, where n is the number of electrons in the electron transfer step, F is Faraday's constant, C is the concentration of ferrocyanide, D is the diffusion coefficient of $[\text{Fe}(\text{CN})_6]^{4-}$ and r is the radius of the electrode.^{16-20, 24}

$$i_{ss} = 4nFDCr \quad (5)$$

In the measurements carried out here we have studied the reduction of $[\text{Fe}(\text{CN})_6]^{3-}$ to $[\text{Fe}(\text{CN})_6]^{4-}$ by *C. vulgaris* using rotating disc electrochemistry (RDE). RDE was chosen for two reasons. Firstly, the constant agitation prevented the algae from settling to the bottom of the flask and secondly, we found that the motion of the electrode helped to reduce biofouling. Amperometry using a rotating disk electrode has previously been used for kinetic analysis of redox reactions in biological systems and is highly reproducible even in cell suspensions.^{25, 26} Torimura *et al.* used RDE to investigate the reduction of 1,4-Benzoquinone (BQ) and 2,6-dimethyl-1,4-benzoquinone (DCBQ) by *Synechococcus* cells.²⁶ When RDE was performed at 0.6 V w.r.t Ag/AgCl, the oxidation current increased linearly under illumination and was constant in the dark. The slope of the increase in the oxidation current was assumed to signify the steady-state rate of electron transfer to the mediators and the reaction followed Michaelis-Menten type kinetics. Kasuno *et al.* also used rotating disk electrochemistry to investigate the kinetics of the photo-induced electron transfer reaction from *Rhodobacter sphaeroides*, to the electron acceptor 2,6-dimethyl-1,4-benzoquinone (DCBQ).²⁵ Using a fixed potential of 0.5 V, optimum for the oxidation of reduced DCBQ, the rate of photo-induced electron transfer was determined by measuring the limiting current for re-oxidation at the rotating disk. Again the reaction was found to follow Michaelis-Menten kinetics.

All the RDE measurements described here were carried out in the dark. In a first set of control experiments *C. vulgaris* was added to a solution containing only ferrocyanide, $[\text{Fe}(\text{CN})_6]^{4-}$, and the oxidation of $[\text{Fe}(\text{CN})_6]^{4-}$ to ferricyanide, $[\text{Fe}(\text{CN})_6]^{3-}$, was measured at the electrode surface. In this experiment the algae could not reduce the $[\text{Fe}(\text{CN})_6]^{4-}$, so the bulk concentration should remain unchanged and the algae should

act as spectator particles in the solution. The purpose of the experiment was to measure the diffusion coefficient of $[\text{Fe}(\text{CN})_6]^{4-}$ in the presence of cells. It has previously been shown that suspended particles (μm sized SiC and calcite particles) can enhance mass transport in electrochemical experiments and give higher than expected diffusion limited currents.^{27, 28} As the algae are 'particles' with diameters in the 5-10 μm range, we postulated that they would also increase mass transfer in RDE experiments as they have similar sizes to the SiC and calcite particles measured previously. If the algae were to enhance mass transport in the same way as the inorganic particles, higher than expected diffusion limited currents would be measured and anomalously high values for the concentration of $[\text{Fe}(\text{CN})_6]^{4-}$ in solution obtained.

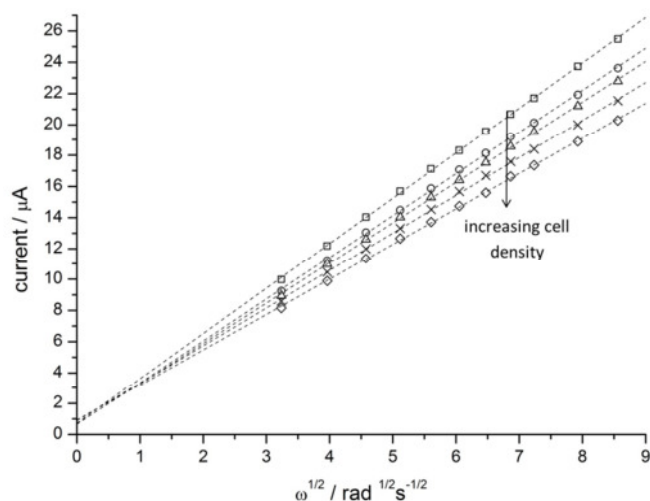


Figure 1. Levich plots showing the effect of increasing cell density on the limiting current as a function of rotation speed. Measurements were carried out at cell densities of 0 (squares), 1.9×10^{10} cells dm^{-3} (circles), 3.8×10^{10} cells dm^{-3} (triangles), 5.7×10^{10} cells dm^{-3} (crosses) and 7.5×10^{10} cells dm^{-3} (diamonds). The intercept was not forced to a specific value when fitting.

Figure 1 shows a Levich plot of the diffusion limited current versus the rotation speed as the number of cells suspended in the electrolyte was increased (the Levich equation is given above, equation 2). The diffusion coefficient of ferrocyanide in the absence of cells (open squares) was calculated to be $6.8 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ in 3N-BBM+V media. The diffusion coefficient is rather temperature dependent; a wide range of experimental values can also be found in the literature at the same temperature, with values between $6.9 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ and $7.6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ being quoted for ferrocyanide at 298K (0.2 mol dm^{-3} KCL_(aq) solution).²⁹⁻³¹

Surprisingly, as cells were added the diffusion limited current decreased. The diffusion limited current in an RDE experiment will decrease if the area of the electrode decreases (see equation 2), for example due to biofouling. In the experiments described here the electrode was cleaned thoroughly between each 3-5

minute measurement (see experimental section for more details). No change in current was seen after cleaning, suggesting that biofouling was not the cause of this effect.

Algae are not solid particles and *C. vulgaris* is surrounded by a polysaccharide cell wall which helps protect the cell from the external environment.^{13, 32, 33} Small molecules can diffuse through the wall so that they can interact with enzymes, such as ferrereductases, in the cell membrane. One likely explanation for the reduction in limiting current is that a fraction of the ferrocyanide is trapped within the algal cell walls or bound to the cell surface. This means that a lower concentration of ferrocyanide remains free in solution to be oxidised at the electrode. The concentration of ferrocyanide calculated to be 'missing' from solution is shown in figure 2; at the highest cell concentration, 14% of the total ferrocyanide concentration would have to be trapped within the cells.

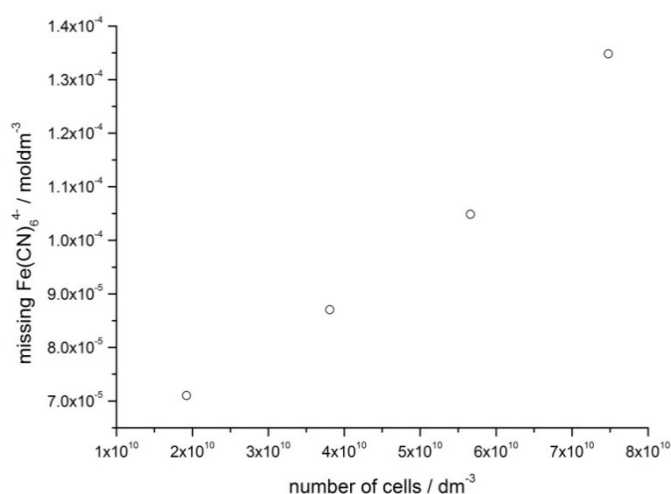


Figure 2. Concentration of missing $\text{Fe}(\text{CN})_6^{4-}$ that would account for the observed changes in the diffusion limited currents (Figure 1).

Sutak *et al* have reported evidence for iron binding to the surface of microorganisms.³⁴ They incubated cells with the ^{55}Fe (ferric citrate or ferrous ascorbate) isotope for 15 minutes, then monitored ^{55}Fe uptake as an excess of ^{56}Fe ('cold iron') was added. In the case of the yeast *Saccharomyces cerevisiae* uptake of ^{55}Fe was substantially reduced by addition of excess cold iron; in contrast uptake of ^{55}Fe by *Thalassiosira pseudonana* continued after dilution with cold iron. Uptake also continued even if the cells were washed with a strong iron chelator and the authors concluded that iron was bound to the cell surface and continued to be available to the cells even after dilution. In total five microalgae species were found to specifically bind large amounts of both ferric and ferrous iron, possibly as a mechanism for concentrating the small amounts of iron available in the marine environment. Evidence for iron adsorption to the surface of two other species of marine phytoplankton was also seen by Hudson *et al*.³⁵ Finally there is evidence that heavy metal ions, such as Cd^{2+} , Zn^{2+} and Cr^{3+} , Cu^{2+} can also be accumulated in algae.³⁶⁻⁴⁰ It has been

suggested, for example, that Cd uptake occurs in two stages: short-term uptake involves physical sorption on the cell surface; and long-term uptake involves intracellular accumulation. Given the evidence that exists for iron and other metal uptake in marine algae, it seems likely that a similar mechanism is operating in the freshwater species of *C. vulgaris* used here.

A second possible explanation is that the 'effective' diffusion coefficient (D_e) of ferrocyanide is reduced by the presence of algae. The diffusion coefficient of free ferrocyanide in solution (D_R) would not change, but D_e would decrease if all ferrocyanide molecules spent some finite amount of time associated with the cells. Given that Sutak *et al* found that iron could not be removed from the marine algae they studied, even when washed with a strong iron chelator, it seems most likely that the first mechanism is operating; however by calculating the effective diffusion coefficient at a given cell concentration and using it in subsequent calculations it allows the decrease in free concentration to be corrected for. The important conclusion is that in our experiments the presence of cells reduced the diffusion limited current substantially.

If a fraction of the reduced redox mediator is spending time trapped on or within the cell wall it has implications for all measurements that quantify ferricyanide (or potentially other metal) reduction by biological cells. Whether the measurements are done electrochemically or colourimetrically, if the redox mediator is trapped within the cell then the experiments will underestimate the concentration of reduced mediator and hence the reducing power of the cells. Concentrations will also be underestimated where biofilms are used, as the redox mediator may be trapped within either the cell wall or extra cellular matrix.⁴¹ A further complication is that when the algal cells are placed in ferricyanide, or mixtures of ferricyanide and ferrocyanide, the ratio of oxidised and reduced mediator trapped within the cells is unknown. One difficulty is that the cells will immediately start to reduce ferricyanide to ferrocyanide and so it is difficult to measure the amount of trapped ferricyanide – hence the approach taken here where trapped ferrocyanide was measured. In order to, as far as possible, correct for the reduction in limiting current in the presence of cells in our experiments, the total bulk ferrocyanide concentration was assumed to remain unchanged and D_e was calculated (Figure 3).

The value of D_e at the appropriate cell concentration was then used to calculate the correct ferrocyanide concentration in further measurements. Care was taken to use these values of D_e only in experiments using the same batch of cells measured over a short timeframe as algal cell walls and the expression of proteins that can interact with redox mediators vary according to cell environment, cell growth conditions and cell age.

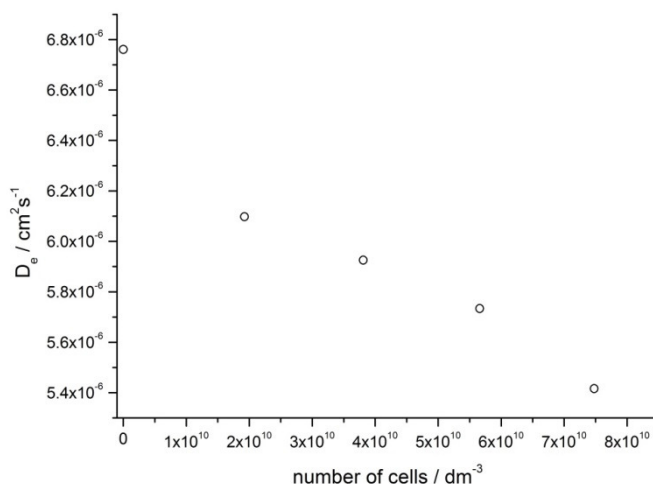


Figure 3. The 'effective' diffusion coefficient of ferrocyanide as a function of algal cell concentration.

In a second set of experiments the reduction of ferricyanide by *C. vulgaris* was measured by RDE. The amount of ferrocyanide being produced by *C. vulgaris* as a function of both ferricyanide concentration and cell concentration was studied. Koutecky–Levich plots, where the background corrected data was plotted according to equation 1, are shown in the supporting information (Figure S3). Each set of experiments was repeated three times and the error bars show one standard deviation. The standard deviation was small at higher ferricyanide and cell concentrations, but larger at the lowest ferricyanide concentrations (3–6 mmol dm⁻³). A good linear fit ($R^2 = 0.9999$) in the Koutecky–Levich plots was found at the higher ferricyanide and cell concentrations. At lower concentrations there was some deviation at the lowest rotation speeds (see S2), but a reasonable fit was still obtained (see S2).

Figure 4 shows the total ferrocyanide being produced by the algae as a function of ferricyanide concentration in solution at cell concentrations between 1.15×10^8 cells mL⁻¹ and 9.26×10^7 cells mL⁻¹. To allow for small changes in cell concentration as aliquots of ferricyanide were added, the data has been normalised to 10^6 cells. The increase in ferrocyanide (corrected for the missing fraction) was approximately linear with increasing ferricyanide in solution, although the gradient appeared shallower at the highest concentrations. As outlined above, where the electrochemical signal is diffusion limited it is difficult to obtain kinetic information about the rate of ferrocyanide turnover. Koutecky–Levich plots do allow kinetic limited currents to be separated from diffusion limited currents if a positive intercept is observed (the current at infinite rotation speed when diffusion is not rate limiting). In the data obtained here a good fit was obtained with an intercept of zero.

Repeat measurements taken at the very lowest concentrations (Figure S3(a)) displayed larger standard deviations as the changes in ferricyanide concentration were small. The data in Fig. S3(a) could have been fit with a slight negative intercept,

but given the larger standard deviation, an intercept of zero was maintained in line with the higher concentration data shown in Fig S3(b) and (c). The ferricyanide turnover was also measured as a function of cell concentration. Figure 4(b) shows the change in ferrocyanide concentration per 10^6 cells. The raw data showed an increase in ferrocyanide as the number of cells increased, however, when normalised to 10^6 cells, it can be seen that the number of ferrocyanide molecules produced *per cell* only increased slightly with cell concentration.

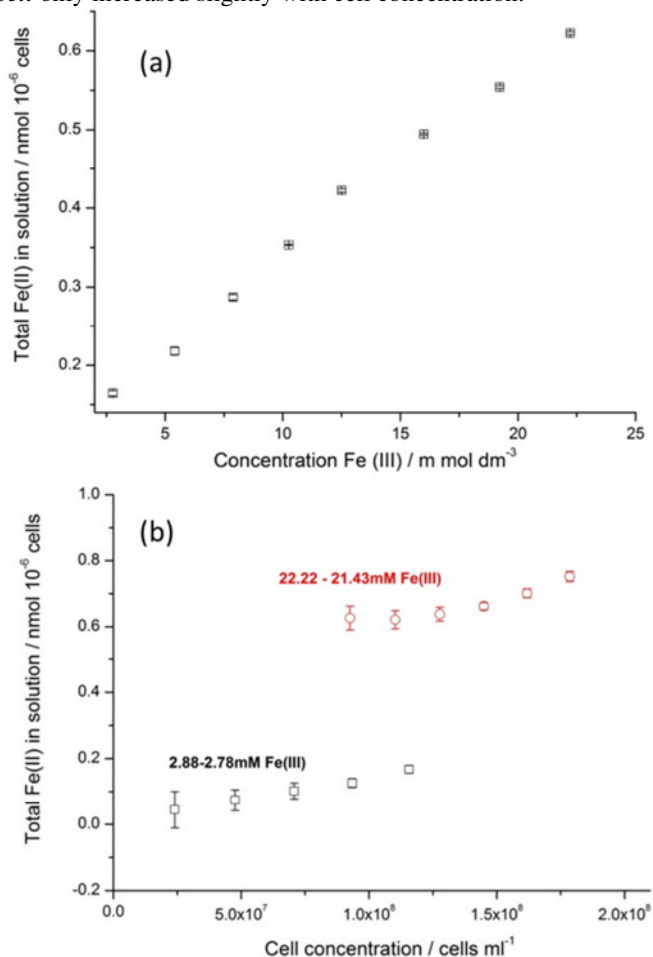


Figure 4(a). The concentration of ferrocyanide produced by 10^6 *C. vulgaris* cells as the substrate (ferricyanide) concentration was increased. Figure 4(b). Total ferrocyanide produced per 10^6 cells when the cell concentration was increased. In both cases the error bars show the error calculated from the standard error of the slope of the linear Koutecky-Levich fits.

The electrochemical detection of ferrocyanide by RDE was compared to electrochemical detection at a stationary FTO macroelectrode (1.33 cm^2 disc). The electrode was clamped at the bottom of a cylindrical container and cells were allowed to settle directly on its surface. The turnover of ferrocyanide back to ferricyanide was measured chronoamperometrically at 100 mV positive of the oxidation potential for ferrocyanide (Figure S3). Figure 5(a) shows the photocurrent (excess current obtained under illumination by red light) observed as a function of both cell concentration and ferricyanide concentration.

Figure 5(b) shows the diffusion limited turnover rate (simply calculated from the steady state oxidation current) as a function of ferricyanide and cell concentration. Figure 5(a) shows a maximum in photocurrent generated at a ferricyanide concentration of 3 mmol dm^{-3} . The reduction of ferricyanide by algae is complicated by the fact that there are differences in the reduction pathways in the dark and under illumination. There is evidence, for example, that ferricyanide reduction in *Chlamydomonas* interacts with both respiration and photosynthesis.⁴² Illumination has been found to increase the rate of iron reduction in iron deficient *Chlorella kessleri*⁴ and iron sufficient and deficient *Chlamydomonas reinhardtii*^{1, 5}, although the increase was much greater in the iron deficient case¹. In these experiments there was a clear increase in currents measured under illumination at the highest cell concentration, meaning that the amount of ferricyanide reduced by the iron sufficient *C. vulgaris* increased under illumination. Chen *et al* also observed a maximum in photocurrent for the reduction of p-benzoquinone by the green microalgae *Tetraselmis subcordiformis*.⁴³

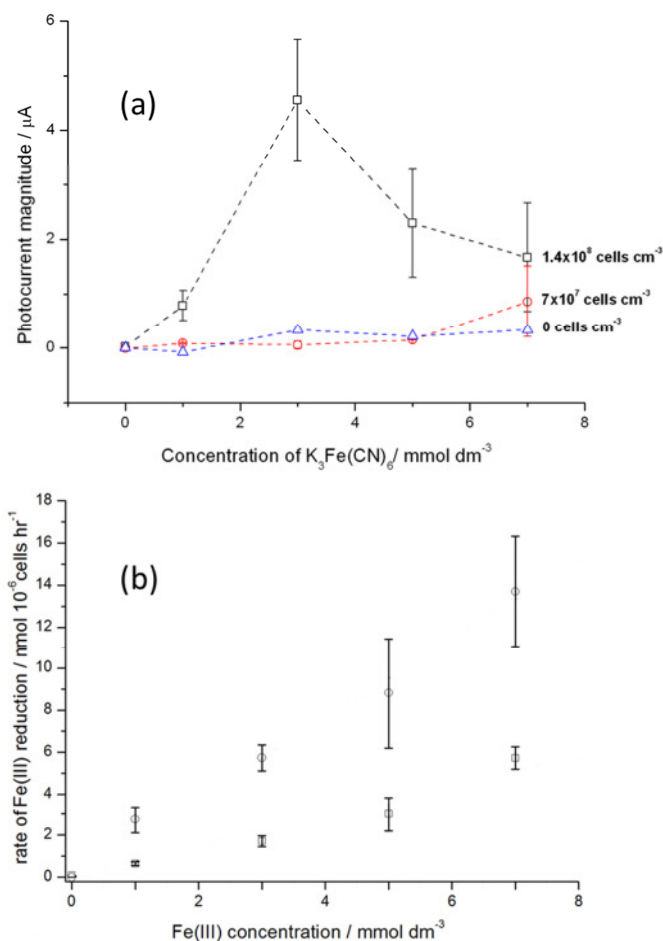


Figure 5(a). The photocurrent generated (measured under red light with a wavelength of 620 nm) with different cell concentrations. Figure 5(b). Calculated rate of ferricyanide reduction in the dark, normalised for 10^6 cells. The squares

were measured for a solution containing 7×10^{10} and the circles for a solution containing 1.4×10^{11} cells dm^{-3} .

Figure 5(b) is interesting; in common with the RDE experiments it shows an increase in current due to the oxidation of ferrocyanide (and hence turnover) at the electrode as the concentration of ferricyanide in the solution was increased. However, a marked increase in current per 10^6 cells is also seen when the cell concentration was increased. In the RDE experiment increasing the cell concentration only led to a small increase in ferrocyanide concentration per 10^6 cells. The most likely explanation for this observation is that the algae settled directly on the electrode surface at the bottom of the electrochemical cell, bringing trapped ferri/ferrocyanide with them. At higher cell concentrations more cells settle on the surface which increases the concentration of ferrocyanide close to the electrode and hence increases the current. Settling out of cells on the electrode was observed by eye in the non-stirred chronoamperometric measurements; the data provides further support for the observation that a significant fraction of ferri/ferrocyanide can be trapped at the surface of the cells.

Conclusions

The presence of *C. vulgaris* cells as ‘spectator particles’ led to a marked reduction in the diffusion limited current for ferrocyanide oxidation. The reduction was attributed to the trapping of ferro/ferricyanide molecules either at the surface or within the cell wall of the algal cells. The result has important implications for measurements used to monitor the reducing power of whole cells or biofilms, including electrochemical and colourimetric techniques. Failure to allow for the adsorption of the mediator onto the cells will lead to an underestimate of the redox mediator present in the solution and hence the total concentration of the reduced species. RDE gave reproducible results for the amount of ferricyanide reduced by *C. vulgaris*. Chronoamperometry at a macroelectrode gave less reproducible currents and the response was influenced by the settling of algae on the electrode surface. The results support the suggestion that ferri/ferrocyanide is trapped within the cells as the settling of cells onto the electrode appeared to increase the local ferrocyanide concentrations and to give an increased current with cell concentration.

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Notes and references

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